APPLICATION IN

THE UNITED STATES
PATENT AND TRADEMARK OFFICE

FOR
METHOD FOR SOLUTION BASED DIAGNOSIS

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Attorney's Docket No. 05033.0003.00US00

METHOD FOR SOLUTION BASED DIAGNOSIS

This application claims benefit to a foreign application, EP 02017313.4, filed August 1, 2002.

FIELD OF THE INVENTION

This invention relates to methods for performing diagnosis of medically relevant conditions by detecting the levels of relevant markers characteristic for the medically relevant condition and the levels of normalization markers. The methods pertain to characterization of the sample in a solution phase, without relying on morphological cell based information.

BACKGROUND OF THE INVENTION

The diagnosis of a large number of medically relevant conditions is currently performed using molecular markers as tools. The molecular tools are generally used as one aspect in a complex examination, taking into account a series of different parameters characterizing the samples to be examined.

In medically relevant analysis, the morphological examination of samples by cytological or histological means is in common use. Such methods based on morphological characterization of cell based samples are applicable for example in analysis of clinical samples such as body fluids, blood, surgical resections, secretions, swabs or lavages.

In screening for cervical cancer, for example, swabs are used for detection of neoplastic lesions of the cervix uteri. In the screening procedure, lesions of different origin have to be distinguished. Causes for lesions may for example be inflammations (due to infectious agents or physical or chemical damage) or preneoplastic and neoplastic changes. In morphological examinations the lesions of different characteristics are sophisticated to distinguish. Thus, for examination of swabs, cytologists and pathologists have to be especially trained and even experienced examiners have a high inter- and intra-observer variance in the assessment of a diagnosis based on cytological specimens. In general the result of the examination is based upon the subjective interpretation of diagnostic criteria by the examining pathologist/cytologist. As a result the rate of false positive and false negative results in the screening tests remains unsatisfying high.

Therefore, in many cases these cytological or histological examination procedures are supported by the use of molecular markers. Such markers are often used in immuno-histochemical staining reactions, or in the course of in-situ hybridization reactions. In the prior art combinations of morphological examinations and immuno-histochemical staining reactions based on marker molecules, characteristic for different medically relevant states of tissues or cells, may lead to enhanced results. The morphologic examination remains laborious and time consuming and thus expensive, even when supported by the molecular methods, that make the results more reliable. Additionally, the diagnosis on a morphologically cell based level is, even when supported by molecular parameters, subject to individual perception of the morphology by individual examiners. Thus the diagnosis is dependent on the person, that performs the examinations.

Only in very few cases, molecular markers may be used as diagnostic tools without further support by cell based morphological examinations. This is especially the case, if markers are to be detected in an environment, where they do only occur under exactly defined conditions. So the methods for diagnosis of conditions on a molecular level only, without the support of cell based information, are restricted to cases, where there are suitable markers, that are non-ambiguously specific for the condition to be characterized. For example, detection of viral infections may be carried out in solutions of samples, because the markers characteristic for the presence of viruses in tissues do not occur in unaffected human tissues.

The reproducibility of the results of examination can be enhanced by the use of supporting molecular tools. However, the problem with the preservation and preparation of the samples may not be overcome by just additionally using molecular markers.

When using molecular tools in cytological or histological examinations, strict precautions in preserving the samples have to be taken to prevent artefacts and improper results of the tests. This is in part due to the instability of the cell based morphological information and in part to the instability of the molecular markers to be detected during the tests. If the samples are not prepared, transported or stored in the appropriate manner, the cell based information, or even the molecular information may get lost, or may be altered. So the diagnosis may be impossible, or may be prone to artefacts. For example, the interpretation of biopsies or cytological preparations is frequently made difficult or impossible because of damaged

(physically or biochemically) cells. Regarding tissue samples or biopsies, the preservation of molecular constituents of the samples, which are subject to a rapid turnover, seems sophisticated due to the time elapsed until penetration of the total sample by appropriate preservatives.

The morphologically supported diagnostic methods performed routinely in the art show two major disadvantages. First, the methods are highly dependent on individual perception of the examiners. Secondly the morphological information is quite sensitive to decay processes and thus may cause artefacts after preparation of the samples. Both aspects contribute to improper reproducibility of the results.

For improved diagnosis of medically relevant conditions, methods that do not depend on cell based morphological information would be desirable.

SUMMARY OF THE INVENTION

The present invention is directed to a method for diagnosing a medically relevant condition of a patient. The method comprises the steps of: obtaining a raw sample containing cells or cell debris from a patient; preparing a sample solution from the raw sample; detecting the levels of one or more relevant markers characteristic for said medically relevant condition in said sample solution; detecting the levels of one or more normalization markers; normalizing the detected level of the relevant markers with respect to said normalization parameters; and diagnosing the medically relevant condition from the normalized levels of said relevant markers within the sample solution. The normalization markers are characteristic for at least one of the following normalization parameters: the presence or absence of a particular cell type among the cells represented within the sample solution, the presence or absence of a particular differentiation pattern in the cells represented within the sample solution, and the presence or absence of particular proliferation properties of the cells represented within the sample solution.

In one embodiment of the invention, the medically relevant condition is a cell proliferative disorder, cancer or a precursory lesion.

The present invention is also directed to a test kit for diagnosing a medically relevant condition.

BRIEF DESCRIPTION OF THE DRAWINGS

The patent of application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Figure 1 shows the specific immunohistochemical staining of endocervical and ectocervical epithelial cells in cervical sections. Figure 1A shows a positive reaction detected in columnar epithelium of the endocervix using an antibody directed against cytokeratin 18 (CK18). Figure 1B shows no specific staining in columnar epithelium of the ectocervix using an antibody directed against cytokeratin 18 (CK18). Figure 1C shows no specific staining in columnar epithelium of the endocervix using an antibody directed against cytokeratin 10/13 (CK10/13). Figure 1D shows a strong staining of the squamous epithelium of the ectocervix using an antibody directed against cytokeratin 10/13 (CK10/13).

Figure 2 shows the Western Blot analysis of solubilized samples from cervical swabs. The numbers 1 to 4 refer to samples (cervical swabs) obtained from individual patients

Figure 3 shows the Western blot and ELISA analysis to demonstrate sample adequacy. Samples of four patients with high-grade cervical dysplasias (see Diagnosis) were analysed using western blot analysis (upper panel of figure). The lower panel of this figure shows the results of ELISA analysis.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides methods for improved diagnosis of medically relevant conditions by solution-based biochemical testing procedures performed in solutions of test samples. The invention provides a method to substitute the cell based morphological information contained within the cytological and/or histological data of the test sample by molecular information obtainable from the solution, wherein the original test sample is dissolved and thus enables for accurate and reproducible assessment of medically relevant diagnosis from dissolved test samples. The method according to the invention comprises the steps of determining the levels of one or more markers associated with the condition to be

diagnosed, determining the level of a set of normalization markers suitable to substitute the information related to morphological aspects of the sample, that would have enabled or supported diagnosis in a cell based test system, comparing and/or combining the data concerning the levels of said markers and assessing diagnosis of a medically relevant condition.

The present invention discovers that diagnosis of conditions, which is normally (in cell based diagnostic systems) enabled and/or supported by histological and/or cytological examination procedures, may be performed in solutions from raw samples containing various cell types of different characteristics, by a method comprising the steps of obtaining a raw sample, dissolving the sample in an appropriate solute, detecting the level of one or more markers associated with the condition to be diagnosed and additionally one or more normalization markers within the sample solution, normalizing the data correlating to the markers associated with said condition with respect to the data correlating to the normalization markers and diagnosing the presence or absence of a condition in the sample.

The method according to the present invention may for example be applied as a primary screening test in cases, where a cytological, histological or pathological examination is normally performed. Using the present invention one may discriminate, if the condition to be diagnosed may be present in the sample. If the solution based diagnosis gives a negative result concerning a particular condition, a further examination may be omissible. In case of positive results, ascertaining by classically applicable methods may follow. Thus, expensive and time consuming microscopic or other examinations could be avoided by means of an inexpensive rapid primary screening test.

One aspect of the present invention is a method for enhanced diagnosis of medically relevant conditions, wherein the assessment of diagnosis is performed using solutions of lysed raw tissue- or cell-samples. The method for diagnosis disclosed according to the present invention does not rely on morphological parameters but enables for a diagnosis by means of biochemical analysis.

A second aspect of the present invention is a method for characterizing a complex sample in solution by means of molecular markers characteristic for the parameters of interest, thus substituting information, which could otherwise be obtainable from cytological or histological examinations.

A third aspect of the present invention is to provide suitable combinations of markers for the diagnosis of particular conditions of medical relevance in complex samples. The markers for normalization are chosen such that parameters included within the raw sample, that enable or support the diagnosis, which are lost by the dissolution of the sample, may be substituted.

A fourth aspect of the present invention are test kits for performing diagnostic or research studies according to the present invention.

The present invention enables for a rapid and easy assay for diagnosing of conditions in raw samples such as body fluids, swabs, lavages (e.g. bronchio-alveolar lavages, breast ductal lavages, etc.), aspirates (needle-apirates, fine-needle-aspirates) or complex cell- or tissue samples. In general, a problem with raw materials is the presence of a number of different cell-types within the sample and the presence of particular microorganisms and extracellular substances. Thus the raw material contains a mixture of cells and compositions, that is prone to give artefacts as results. The presence of different cell types with different proliferative characteristics, of organisms and substances within the raw sample gives rise to multiple factors, that may contribute to the particular level of a marker molecule. Detecting solely the level of one single molecular marker may thus only lead to a diagnostically useful information, if further (morphological) parameters concerning the raw sample are provided. All morphologic data obtainable from the raw sample are lost due to lysis in solution. Yet there are suitable molecular markers corresponding to particular morphologic or other parameters obtainable by histological, cytological methods.

For example, the information about the single constituents within the raw sample may be classically obtained by microscopic examination. Morphologic inspection gives hints about the differentiation, the localization of cells, as well as about the environment, in which the cells appear. In cytological preparations of cervical-swabs, for example, the particular cells may be identified as epithelial cells and further categorized as e.g. endocervical or ectocervical epithelial cells. Even the presence of non-cervical cells such as endometrial cells may be ascertained easily by microscopic inspection.

According to the present invention, raw materials may directly be dissolved in an appropriate solvent without further preparation or characterization independent of the

homogeneous or heterogeneous character of the sample material. Data, which are lost through lysis of the material are contained within the sample solution encoded by the levels of a series of marker molecules and may thus be reconstructed using said molecular data for normalization to the respective morphologic characteristics. This is achieved by employing a suitable set of molecular markers for each of the characteristic parameters needed for unambiguous diagnosis. By detecting a suitable array of markers one may assess the relevant parameters characterizing the raw sample and thus overcome the disadvantage of loss of information through lysis of the sample.

The testing procedure according to the present invention includes detecting the levels of markers characteristic for cell conditions in question and of markers for normalizing the data with respect to parameters characterizing the particular environment in the test sample. The markers suitable for the present invention may be of various origin. The expression pattern of a marker, that is suitable for the detection of conditions in question, may be dependent on the proliferative status of cells, on the differentiation status, on the cell type or on the organism. Examples for appropriate markers are set forth below.

The term diagnosis as used herein generally comprises any kind of assessment of the presence of absence of a medically relevant condition. Diagnosis thus comprises processes such as screening for the predisposition for a medically relevant condition, screening for the precursor of a medically relevant condition, screening for a medically relevant condition, clinical or pathological diagnosis of a medically relevant condition, etc.. Diagnosis of medically relevant conditions as used herein may comprise examination of any condition, that is detectable on a cytological, histological, biochemical or molecular biological level, that may be useful in respect to the human health and/or body. Such examinations may comprise e.g. medically diagnostic methods and research studies in life sciences. In one embodiment of the invention, the method is used for diagnosis of medically relevant conditions such as e.g. diseases. Such diseases may for example comprise disorders characterized by non-wild type proliferation of cells or tissues.

In one embodiment, the diagnosis pertains to diagnosis of cancers and their precursory stages, to monitoring of the disease course in cancers, to assessment of prognosis in cancers and to detection of disseminated tumor cells e.g. in the course of minimal residual disease

diagnosis. The method according to the present invention may for example be used in the course of clinical or pathological diagnosis of cancers and their precursory stages or in routine screening tests as performed for particular cancers such as for example for examination of swabs e.g. in screening tests for cervical lesions, of bronchial lavages for lung cancer or of stool for lesions of the gastrointestinal tract, e.g. colorectal lesions.

The method according to the present invention is applicable to all kinds of medically relevant conditions.

Medically relevant conditions as used according to the present invention may for example be compositions of tissues, body fluids, secretions, washes or swabs. Such conditions may for example comprise the cellular composition of body fluids, such as the composition of blood, the composition of liquor or the composition of semen. In this context the compositions shall be for example the presence or absence of particular cell types (e.g. pathogens, such as, viruses etc., preneoplastic, neoplastic and/or dysplastic cells etc.), the presence or absence of differentiation patterns of particular cell types, the total number of a particular cell types (e.g. erythrocytes, leucocytes, sperm, etc.), the total number of all cells of any cell types or the fraction of cells of particular other characteristics present or absent in the sample.

Furthermore, medically relevant conditions may also comprise disorders related to cells, or tissues. The conditions to be diagnosed may comprise parameters related to cells in cytological or histological tissue samples. The conditions may comprise a differentiation pattern of cells in a tissue sample, such as surgical resection samples, biopsies, swabs, lavages etc. Such conditions may comprise e.g. congenital disorders, inflammatory disorders, mechanical disorders, traumatic disorders, vascular disorders, degenerative disorders, growth disorders, benign neoplasms, malignant neoplasms. Another aspect of the conditions according to the present invention may comprise conditions characterized by the presence or absence of proliferative characteristics. Conditions characterized by the presence or absence of proliferative characteristics may be for example cell proliferative disorders.

Cell proliferative disorders according to the present invention comprise diseases characterized by abnormal growth properties of cells or tissues compared to the growth properties of normal control cells or tissues. The growth of the cells or tissues may be for example abnormally accelerated, decelerated or may be regulated abnormally. Abnormal

regulation as used above may comprise any form of presence or absence of non wild-type responses of the cells or tissues to naturally occurring growth regulating influences. The abnormalities in growth of the cells or tissues may be for example neoplastic or hyperplastic.

In one embodiment, the cell proliferative disorders are tumors. Tumors may comprise tumors of the head and the neck, tumors of the respiratory tract, tumors of the anogenital tract, tumors of the gastrointestinal tract, tumors of the urinary system, tumors of the reproductive system, tumors of the endocrine system, tumors of the central and peripheral nervous system, tumors of the skin and its appendages, tumors of the soft tissues and bones, tumors of the lymphopoietic and hematopoietic system, etc. Tumors may comprise for example neoplasms such as benign and malignant tumors, carcinomas, sarcomas, leukemias, lymphomas or dysplasias. In a particular embodiment, the tumor is for example cancer of the head and the neck, cancer of the respiratory tract, cancer of the anogenital tract, cancer of the gastrointestinal tract, cancer of the skin and its appendages, cancer of the central and peripheral nervous system, cancer of the urinary system, cancer of the reproductive system, cancer of the endocrine system, cancer of the soft tissues and bone, cancer of the hematopoietic and lymphopoietic system.

Tumors of the anogenital tract may comprise cancer of the perineal, the perinanal and the scrotal skin, cervical cancer, cancer of the vulva, cancer of the vagina, caner of the penis, cancer of the anus, etc. Cervical cancer may comprise squamous lesions, glandular lesions or other epithelial tumors. Squamous lesions comprise, e.g., cervical intraepithelial neoplasias (mild, moderate and severe dysplasia), carcinoma in-situ, squamous cell carcinoma (e.g., keratinizing, nonkeratinizing, verrucous, warty, papillary, lymphoepithelioma-like). Glandular lesions may comprise atypical hyperplasias, adenocarcinoma in-situ, andenocarcinoma (such as, e.g., mucinous, endometrioid, clear cell, adenoma malignum, papillary, serous or mesonephric adenocarcinoma). Other epithelial tumors may comprise adenosquamous carcinoma, glassy cell carcinoma, adenoid cystic carcinoma, adenoid basal carcinoma, carcinoid tumor, small cell carcinoma and undifferentiated carcinoma. For more detailed information, confer "Kurman, R., Norris, H., *et al.*, *Tumors of the Cervix, Vagina, and Vulva, Atlas of Tumor Pathology*, 1992, AFIP," the contents of which shall be incorporated herein by reference.

Gastrointestinal tumors may comprise colon cancer, cancer of the colon ascendens, of the colon descendens, of the colon transversum, of the sigmoidum, of the rectum, cancer of the small intestine, cancer of the jejunum, cancer of the duodenum, gastric cancer, oesophageal cancer, liver cancer, cancer of the bile, cancer of the biliary system, pancreatic cancer, etc. A comprehensive overview over gastrointestinal lesions is given in "Hamilton Sr, Aaltonen LA (Eds.): World Health Organization Classification of Tumours, *Pathology and Genetics of Tumors of the Digestive System*, IARC Press: Lyon 2000," which shall be incorporated herein by reference.

Tumors of the respiratory tract may comprise any malignant condition of the respiratory tract such as, e.g., cancer of the lung, the alveoles, the bronchioles, the bronchial tree and the broncus, the nasopharyngeal space, the oral cavity, the pharynx, the nasal cavity and the paranasal sinus. Lung cancer such as small cell lung cancer, non-small cell lung cancer, squamous cell lung carcinoma, small cell lung carcinoma, adenocarcinoma of the lung, large cell lung carcinoma, adeno-squamous lung carcinoma, carcinoid tumor of the lung, broncheal gland tumor or (malignant) mesothelioma. An overview over tumors of the respiratory tract may be found in Colby TV, *et al.*: *Tumors of the Lower RespiratoryTract,* Atlas of Tumor Pathology, Third Series, Fascicle 13, AFIP: Washington 1995," which shall be incorporated herein by reference.

Tumors of the urinary system may comprise bladder cancer, cancer of the kidney, renal pelvis, cancer of the ureters and cancer of the urethra, etc. Tumors of the reproductive system may comprise cancer and precursory stages thereof of the ovary, the uterus, the testis, the prostate, the epididymis, etc.

In all cases, the methods according to the present invention also apply to precursor stages of the lesions, tumors or cancers.

In one embodiment, the method according to the present invention pertains to the detection of disseminated tumor cells or metastases.

In one embodiment of the invention, the carcinoma is e.g. cervical cancer, colon cancer, gastric cancer, breast cancer, bladder cancer, lung cancer, cancer of the oral cavity etc.

The present invention provides a number of robust, fast and easy ways to preserve molecular properties of samples, whereby the morphological information of samples is lost.

Samples may be e.g. prepared in a reproducible and easy to store and transport form by dissolving the cellular components of the raw sample in a suitable solvent immediately after or even during obtaining the sample. Body fluids may directly be transferred from the body of an individual to a solution containing suitable detergents and preservative substances. Furthermore, tissue samples may immediately be transferred to denaturing lysis conditions (eventually supported by physical forces) and be thus preserved. Using appropriate ingredients in the solvent, the molecular components of the original sample may be preserved, and no degradation may occur. The degradation by enzymatic activities may, for example, be minimized by the use of enzyme inhibitors. Thus, a solution of test samples may easily represent the molecular properties of a test sample at the time of dissolution, without requiring additional preservative precautions.

Raw samples may comprise clinical samples, such as e.g. secretions, swabs, lavages, body fluids, blood, urine, semen, stool, bile, liquor, bone marrow, biopsies, cell- and tissue-samples. Biopsies as used in the context of the present invention may comprise e.g. resection samples of tumors, tissue samples prepared by endoscopic means or punch- or needle- biopsies of organs. Furthermore, any sample potentially containing the marker molecules to be detected may be a sample according to the present invention. In one embodiment of the invention, the sample comprises cervical swabs, bronchial lavages, stool etc. Raw sample as used in the context of the present invention may comprise fixed or preserved cell or tissue samples. E.g. cells preserved in suitable solutions (alcohols etc.) or fixed tissue samples may be used as raw samples in the methods according to the present invention.

A raw sample according to the method of the present invention includes any sample comprising cells or cell debris. The cells may for example be prokaryotic or eukaryotic cells. When the present invention is applied for the detection of infectious diseases, the cells to be determined may be cells of microorganisms such as chlamydia, *E. coli*, candida, etc.

According to the present invention, all or part of the molecular components of the raw samples are solubilized in a suitable lysis buffer comprising e.g. solvents. Such solvents may for example be aqueous solutions of chaotropic agents such as e.g. urea, GuaSCN, Formamid, of detergents such as anionic detergents (e.g. SDS, N-lauryl sarcosine, sodium deoxycholate, alkyl-aryl sulphonates, long chain (fatty) alcohol sulphates, olefine sulphates and sulphonates,

alpha olefine sulphates and sulphonates, sulphated monoglycerides, sulphated ethers, sulphosuccinates, alkane sulphonates, phosphate esters, alkyl isethionates, sucrose esters), cationic detergents (e.g. cetyl trimethylammonium chloride), non-ionic detergents (e.g. Tween 20, Nonidet P-40, Triton X-100, NP-40, Igepal CA-630, N-Octyl-Glucosid) or amphoteric detergents (e.g CHAPS, 3-Dodecyl-dimethylammonio-propane-1-sulfonate, Lauryldimethylamine oxide) and/or of alkali hydroxides such as e.g. NaOH or KOH. The solvent is designed, so that cells, cell debris, nucleic acids, polypeptides, lipids and other biomolecules potentially present in the raw sample are dissolved. The solution for dissolving the raw samples according to the present invention may furthermore comprise one or more agents that prevent the degradation of components within the raw samples. Such components may for example comprise enzyme inhibitors such as proteinase inhibitors, RNAse inhibitors, DNAse inhibitors etc. In one embodiment of the present invention the sample is lysed directly in the form it is obtainable from the test-individuals. In another embodiment of the present invention the sample may be further purified before being lysed. Such purification procedures may for example comprise washing away of contaminants such as mucus or the like, separation or concentration of cellular components, preserving and transporting of the cells. Thus the cellular components of the raw samples are included in a single sample solution.

The preparation of a sample for use in a method as disclosed herein may also comprise several steps of further preparations of the sample, such as separation of insoluble components, isolation of polypeptides or nucleic acids, preparation of solid phase fixed peptides or nucleic acids or preparation of beads, membranes or slides to which the molecules to be determined are coupled covalently or non-covalently.

According to the present invention, the detection of the marker molecules is performed directly from this solution. The detection may be carried out in solution or using reagents fixed to a solid phase. In certain embodiments of the present invention the detection of the marker molecules is performed from a solution of dissolved body samples. Therefore detection may be carried out in solution or using reagents fixed to a solid phase. A solid phase as used in the context of the present invention may comprise various embodiments of solid substances such as planar surfaces, particles (including micro-, nano-particles or even smaller particles). In certain embodiments particles may be provided as beads, colloids or the like. The fixation of reagents

to the solid phase in a test kit or an in-vitro diagnostic device may be effected via direct fixation or via indirect fixation. Direct fixation may e.g. be effected by covalent or non-covalent binding or association to surfaces. Indirect fixation may be effected through binding of the reagents (e.g. antibodies, probes etc.) to agents which themselves are directly fixed to solid phases. Such agents may comprise antibodies or other binding agents like avidin, streptavidin, biotin or the like. The detection of one or more molecular markers may be performed in a single reaction mixture or in two or more separate reaction mixtures. The detection reactions for several marker molecules may for example be performed simultaneously in multi-well reaction vessels or as the case may be on one single or two or more separate test strips. The markers characteristic for the cell proliferative disorders may be detected using reagents that specifically recognise these molecules. Simultaneously the normalization markers may be detected using reagents, that specifically recognize them. The detection reaction for each class of markers may comprise one or more further reactions with detecting agents either recognizing the initial marker molecules or preferably recognizing the prior molecules (e.g. primary antibodies) used to recognize the initial markers. The detection reaction further may comprise a reporter reaction indicating the level of the markers characteristic for cell proliferative disorders or the normalization markers.

The terms "marker" or "marker molecule" in all their grammatical forms as used in the context of the present invention refers to nucleic acid as well as polypeptide molecules. Marker or marker molecule thus comprises e.g. RNA (mRNA, hnRNA, etc.), DNA (cDNA, genomic DNA, etc.), proteins, polypeptides, proteoglycans, glycoproteins and the respective fragments of these molecules. The term "relevant marker" shall refer to marker molecules characteristic for a medically relevant condition. The term normalization marker shall refer to marker molecules used for normalization purposes.

A level of a marker molecule as used herein refers to a semiquantitave as well as a quantitative value regarding the amount of the respective marker present in a sample. A quantitative value may e.g. be represented in terms of a concentration. A semiquantitative value may be expressed in terms of a scale of levels e.g. undetectable levels, low levels, intermediate levels, high levels or any other suitable mode. The level of a marker may also be represented in terms of a dependent parameter such as the intensity of a signal generated in an assay format in

response to the presence of a marker molecule.

A probe for the detection of the marker molecules as used in the context of the present invention shall be any molecule, that specifically binds to said marker molecules. The probe may for example be an antigen binding agent such as antibodies (monoclonal or polyclonal), antibody fragments or artificial molecules comprising antigen binding epitopes, DNA or RNA binding molecules such as proteins or nucleic acids. Nucleic acids binding to other nucleic acids may for example be peptide nucleic acids (PNAs) or oligonucleotides (RNA, DNA, PNA, artificial nucleic acids, etc.) for detection purposes or primers.

A molecule is said to recognize another molecules if it specifically interacts with that molecule. Specific interaction may for example be specific binding to or of the other molecule.

The reporter reaction may be for example a reaction producing a colored compound. In one embodiment of the present invention the reporter substances correlated to the particular markers develop different colors. In another embodiment, the normalization marker specific reporter may be a molecule quenching the signal produced by the reporter molecule specific for the marker, characteristic for the medically relevant condition, in dependence on the level of the normalization marker present in the sample. In yet another embodiment the reporter reactions may produce fluorescent dyes with differing wavelength characteristics. In a further embodiment of the present invention the reporter reaction may comprise light emitting reactions with different wavelength characteristics for the reporter substances specific for either marker to be detected. In another embodiment of the present invention the reporter reaction may comprise the emission of radioactive radiation and additional methods for visualizing or quantifying the radiation. In one embodiment, the different marker molecules may be recognized by agents, that bear radio-nuclides emitting radiation with different energetic properties, so that the signals referring to marker molecules could be distinguished.

Applicable formats for the detection reaction according to the present invention may be blotting techniques, such as Western-Blot, Southern-blot, Northern-blot. The blotting techniques are known to those of ordinary skill in the art and may be performed for example as electro-blots, semidry-blots, vacuum-blots or dot-blots. Furthermore immunological methods for detection of molecules may be applied, such as for example immunoprecipitation or immunological assays, such as EIA, ELISA, RIA, lateral flow assays, flow through assays,

immunochromatographic strips, etc. Immunoassays for use in the invention may comprise competitive as well as non-competitive immunoassays such as sandwich assays.

In certain embodiments of the invention immunochemical or nucleic acid based testing may be performed using a testing device for clinical laboratories. Such testing device may comprise any device suitable for immunochemical or nucleic acid based testing including any format such as e.g. Point of care testing devices as well as bench top or laboratory devices. The devices may be e.g. provided as open or closed platform systems. The system may be based on any suitable methodology such as e.g. employing microtiter plates, multiwell plates, flow through or lateral flow systems, microchip or array based systems or bead or membrane based systems. The detection methods employed may comprise any methods known to those of skill in the art useful for immunochemical or nucleic acids based detection reactions. Such detection systems may be e.g. luminescence systems (electroluminescence, bioluminescence, photoluminescence, radioluminescence, chemiluminescence, electrochemoluminescence), fluorescence based systems, conductivity based detection systems, radiation (light, UV, X-ray, gamma etc.) or any other known method.

The method for detection of the level of the marker molecules in one embodiment of the present invention is any method, which is suited to detect even very small amounts of specific molecules in biological samples. Furthermore any method for detection of the marker molecules irrespective of the sensitivity may be applied. The detection reaction according to the present invention may comprise for example detection reactions on the level of nucleic acids and/or detection reactions on the level of polypeptides. In one embodiment of the invention, the detection of the marker molecules may comprise the detection of particular splicing variants. In another embodiment of the present invention, the detection method may comprise the detection of modifications of marker molecules such as phosphorylation or glycosylation etc of polypeptides or the methylation of nucleic acid molecules in samples.

In one embodiment of the invention, the detection of the level of marker molecules is carried out by detection of the level of nucleic acids coding for the marker molecules or fragments thereof present in the sample. The means for detection of nucleic acid molecules are known to those skilled in the art. The procedure for the detection of nucleic acids can for example be carried out by a binding reaction of the molecule to be detected to complementary

nucleic acid probes, proteins with binding specificity for the nucleic acids or any other entities specifically recognizing and binding to said nucleic acids. This method can be performed as well in vitro as directly in-situ for example in the course of a detecting staining reaction. Another way of detecting the marker molecules in a sample on the level of nucleic acids performed in the method according to the present invention is an amplification reaction of nucleic acids, which can be carried out in a quantitative manner such as for example the polymerase chain reaction. In one embodiment of the present invention e.g. real time RT PCR may be used to quantify the level of marker RNA in samples of cell proliferative disorders.

In another embodiment of the invention, the detection of the level of marker molecules is carried out by determining the level of expression of a protein. The determination of the marker molecules on the protein level may for example be carried out in a reaction comprising a binding agent specific for the detection of the marker molecules. These binding agents may comprise for example antibodies and antigen-binding fragments, bifunctional hybrid antibodies, peptidomimetics containing minimal antigen-binding epitopes etc. The binding agents may be used in many different detection techniques for example in western-blot, ELISA, RIA, EIA, flow through assay, lateral flow assay, latex-agglutination, immunochromatographic strips or immuno-precipitation. Generally binding agent based detection may be carried out as well in vitro as directly in situ for example in the course of an immunocytochemical staining reaction. Any other method suitable for determining the amount of particular polypeptides in solutions of biological samples can be used according to the present invention.

Methods for the detection of the modified states of nucleic acid molecules and/or polypeptides are known to those of ordinary skill in the art.

Methods for detection of methylation of nucleic acids are known to those of skill in the art and may comprise for example methods employing chemical pre-treatment of nucleic acids with e.g. sodium bisulphite, permanganate or hydrazine, and subsequent detection of the modification by means of specific restriction endonucleases or by means of specific probes e.g. in the course of an amplification reaction. The detection of methylation may furthermore be performed using methylation specific restriction endonucleases. Methods for the detection of methylation states in nucleic acids are e.g. disclosed in patent application EP02010272.9,

US5856094, WO0031294, US6331393 etc. The cited documents are incorporated herein by reference.

Detection of modified states of polypeptides may for example comprise binding agents specifically recognizing modified or unmodified states of polypeptides. Alternatively enzymes such as phosphatases or glycosylases may be used to remove modifications in molecules. The presence or absence of modifications can thus be detected by determination of mass or charge of the molecules by means of electrophoresis, chromatography, mass spectrometry etc. prior and subsequent to the incubation with a respective enzyme.

In a further embodiment of the present invention, the detection of a series of marker molecules is carried out on the level of polypeptides and simultaneously the detection of a further series of marker molecules and/or of all or some of the same marker molecules is carried out on the level of nucleic acids.

Markers associated with medically relevant cellular conditions may e.g. be molecules which influence and/or reflect the proliferation and/or differentiation characteristics of cells and/or tissues. Such molecules may comprise for example cell cycle regulatory proteins, proteins associated with the DNA replication, transmembrane proteins, receptor proteins, signal transducing proteins, calcium binding proteins, proteins containing DNA-binding domains, metalloproteinases, kinases, kinase inhibitors, chaperones, embryogenesis proteins, heat shock proteins or enzymes which modify other proteins posttranslationally thus regulating their activity, or nucleic acids coding for the named proteins. Also mRNA coding for the named proteins may be marker molecules useful according to the present invention. In one embodiment the marker associated with the cell proliferative disorder may be for example uniquely expressed in cells affected by the disorder, may be not expressed in said cells or may be overexpressed in said cells.

Marker molecules for use according to the present invention may comprise one or more markers chosen from p13.5, p14, p15, p16 (also referred to $p16^{INK4a}$), p19, p21, p27, p53, pRb, p14ARF, cyclin A, cyclin B, cyclin E, MDM-2, MCM2, MCM5, MCM6, CDC2, CDC6, Id1, osteopontine, GRP, renal dipeptidase, her2/neu, TGFßII receptor, HPV associated markers e.g. derived from HPV genes L1, L2, E1, E2, E4, E5, E6 or E7, etc. A selection of markers useful

in one embodiment of the present invention for the detection of medically relevant conditions is shown below in Table 1.

In one embodiment the marker for a medically relevant condition may be a marker for tumors (tumor markers). The marker molecules characteristic for tumors may e.g. be proteins, that are expressed in a non-wild type manner in tumors compared to normal control tissue. Non-wild type expression as used herein may comprise increased or decreased levels of expression or lack of expression or expression of non-wild type forms of the respective molecules. Expression of non-wild type forms of a protein may comprise expression of mutated forms of proteins, arising by insertion, deletion, substitution, or frameshift mutations or any other known types of mutations in proteins or nucleic acids. In all cases of the expression of non-wild type proteins or non-wild type levels of proteins the proteins, polypeptides or fragments thereof or nucleic acids encoding these proteins or polypeptides or fragments of these nucleic acids may be used as molecular markers associated with tumors and may thus be understood under the term "tumor marker" as used in the context of the present invention. Proteins that show non-wild type expression in association with tumors are disclosed for example in the documents WO9904265A2, WO0149716A2, WO0055633A2 and WO0142792A2, which shall be incorporated by reference herein.

In one embodiment of the invention, the marker characteristic for the medically relevant condition may be a cell cycle regulatory protein such as for example a cyclin, a cyclin dependent kinase or a cyclin dependent kinase inhibitor. In a further embodiment of the invention the marker characteristic for the medically relevant condition may be a marker associated with a transient or a persistent viral infection. The viral infection may comprise an infection by a human papilloma virus (HPV) such as high risk or low risk HPV. The high risk HPV may comprise HPV subtypes such as e.g. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58. The markers for HPV infection may e.g. comprise HPV expression products of HPV genes L1, L2, E2, E4, E5, E6 or E7. In a third embodiment of the invention a marker characteristic for a viral infection may be used in combination with any other marker for a medically relevant condition such as e.g. in combination with a cell cycle regulatory protein. Combinations of marker molecules, which may be of special interest with respect to HPV association are e.g. disclosed in WO0208764 which document shall be incorporated herein by reference.

In one embodiment, cell cycle regulatory proteins for use in combination with HPV markers may for example be chosen from a group comprising pRb, p53, p14 ARF, cyclin dependent kinase inhibitors. In one special embodiment for example $p16^{INK4a}$ may be used in combination with markers for HPV infection (e.g. L1, L2, E2, E4, E5, E6 or E7).

It must be understood, that as the case may be markers useful as markers for medically relevant conditions in certain embodiments may serve as markers for normalization in certain other embodiments and vice versa. However, in each single embodiment, a marker can only serve either as marker for the medically relevant condition or as marker for normalization. For example, Ki67 as a marker for cell proliferation may be useful as a normalization marker in certain embodiments (e.g. in combination with p16, p14ARF, claudin-1 or others as markers for medically relevant condition). In other embodiments, Ki67 may serve as a marker for medically relevant condition (e.g. as a marker for cervical dysplasia or other dysplastic diseases) in combination with suitable normalization markers (e.g. cytokeratins, catenins or others). Various other markers may likewise serve either as a marker for medically relevant conditions or for a normalization depending on the particular embodiment of application.

Normalization markers according to the present invention may comprise for example housekeeping genes, such as actin, gapdh, histone proteins, phospholipase, ß2-microglobulin, proteins associated with active cell proliferation such as e.g. Ki67, PCNA or statin, or proteins characteristic for particular cell types such as for example CK20 in epithelial cells or any cell specific cell-surface antigens. In addition, carbohydrate structures present on glycoproteins, proteoglycans, lectin receptors such as the concanavalin A receptor, mucins and enzymes which are involved in the biosynthesis of these molecules such as GalNac transferases and oligosaccharyltransferases might also serve as normalization markers. The type of marker protein has to be chosen according to the information, which shall be provided by the marker. Principally the markers useful for particular medically relevant conditions may under certain circumstances be useful as normalization markers. A selection of markers useful in performing the methods according to the present invention are given in Table 1.

As well concerning markers for medically relevant conditions as well as concerning normalization markers modified states of molecules (such as polypeptides and nucleic acids) may be used as markers in the method according to the present invention. For example

phosphorylated, glycosylated or otherwise modified polypeptides or methylated nucleic acids may be addressed as markers in the method according to the present invention.

Normalization as used according to the present invention shall comprise any method suitable for relating the detected levels of markers to parameters valuable for the assessment of the diagnosis. One aspect of this normalization may be a reconstruction of the relevant cytological and histological information contained within the raw sample by means of suitable molecular markers detectable in the sample solutions. Normalization may comprise for example the detection of the total number of cells present in the sample, of the presence or absence of a particular cell types in a sample, of the presence or absence of an organism or of cells of an organism in a sample, of the number of cells of a particular cell type or organism present in the sample, of the proliferative characteristics of cells present in the sample or of the differentiation pattern of the cells present in the sample.

In certain embodiments normalization may also comprise proving the adequacy of the test, wherein as the case may be inadequate test results may be discarded or classified as invalid. Therefore ,normalization as used in the context of the present invention may comprise qualitative or semi-quantitative methods for normalization. In certain embodiments, semi-quantitative normalization may comprise determining a threshold value for a normalization marker. In one embodiment, semi-quantitative normalization may be applied e.g. as follows: the level determined for the relevant marker may be regarded as a valid test result only if the level of the normalization marker exceeds a defined threshold value; in case the threshold value is not reached the test result for the relevant marker is regarded as invalid; diagnosis may not be assessed on the basis of the test. In other embodiments a threshold may be set that may not be exceeded. In certain embodiments, qualitative normalization may be performed with respect to the presence or absence of a normalization marker. In those cases, e.g. the value determined for the relevant marker is compared to the presence or absence of a normalization marker. As predefined, the value is valid only in case the normalization parameter (presence or absence of a detectable level of the normalization marker) is met.

Table 1

marker for	cell type	antigen	Antibody	supplier	Literature
cell type	epithelial cells	human epithelial cell surface glycoprotein	HEA125 IgG1 (W, IHC, ICC, IF)	Research Diagnostics Inc.	Kommoss et al., Hum Pathol. 2000 Sep;31(9):1055-61
		Human epithelial proliferation 40 kD protein (from LoVo)	AUA-1 IgG1 (Elisa)	Research Diagnostics Inc.	Gottschalk et al, Pathol Res Pract. 1992 Feb;188(1-2):182-90
		Human epithelial antigen (34+39 kD)	Ber-EP4, IgG1 (IHC, Elisa)	Dako	Latza U et al., J Clin Pathol. 1990 Mar;43(3):213-9
		Human epithelial proliferating antigen (40 kD)	AUA-1 (Elisa, W, IHC)	Research Diagnostics Inc.	Epenetos, A et al., Lancet. 1982 Nov 6;2(8306):1004-6
	endocervix columnar cells	Cytokeratin 18 (45 kD)	RGE 53, IgG1 (W, IHC, IF)	Research Diagnostics Inc.	Smedts F et al., Am J Pathol. 1990 Mar;136(3):657-68
		Cytokeratin 18 (45 kD	RCK 106 (W, IHF, IHC)	Research Diagnostics Inc	Smedts F et al., Am J Pathol. 1990 Mar;136(3):657-68
		Cytokeratin 8 (52.5 kD)	CAM 5.2 (W, IHC)	BD PharMingen	Smedts F et al., Am J Pathol. 1990 Mar;136(3):657-68
	Endocervical columnar cells	Mucin Antigens (Tn, STn, MUC1, MUC2	DF3	Centocor	Tashiro et al., Hum Pathol. 1994 Apr;25(4):364-72
	Endocervic Columnar cells	Concanavalin A receptor			Herckenrode et al., Br J Cancer. 1988 Mar;57(3):293-4 ; Koch et al., Br J Cancer. 1986 Jan;53(1):13-22
	Endocervix	GalNacTransferase Oligosaccharyltransferase			Chilton et l., Endocrinology. 1988 Sep;123(3):1237-44

	Endocervic/ Ectocervix	Lectins (ConA, WGA, PNA, UEA I, DBA, SBA, SNA			Di Loretto et al., Basic Appl Histochem. 1987;31(2):143-52; Versura et al., Basic Appl Histochem. 1988;32(2):219-27
	ectocervix squamous cells	Plakophilin (80.5 kD	PP1-5C2, IgG1 (W, Elisa, IHC, IF)	Research Diagnostics Inc.	Heid, HW, Differentiation. 1994 Dec;58(2):113-31
	endometrial cells	Vimentin	VIM 3B4, IgG1, (W, ELISA, IF, IHC)	Research Diagnostics Inc.	Smedts F et al., Am J Pathol. 1990 Mar;136(3):657-68
	Erythrocytes	Haemoglobin	RDI-CBL63 , IgM (RIA,EIA)	Research Diagnostics Inc.	Smith et al., J. Histochem. Cytochem. 1998
Inflamm -ation	neutrophilic granulocytes NK-cells Macrophages	CD16(NK, Macro, Gran)	DJ130-c, IgG1 (IHC)	DIANOVA	Grundhoever D and Patterson BK, Cytometry 2001;46:340-344
		CD56(NK)	clone Ki-M6		Hermann et al., J. Clin. Immunol. 1990
		CD68(Macro)	(antiCD68)		Cavayal et al., Eur. J. Immunol: 1998(6)1991-2002 (CD56)
	B-cells	CD19 (CD20)	clone AE 1, FACS	DIANOVA	Harrada et al., Blood 1993 ;81 :2658-63 (CD19) Mason et al., Am J. Pathol1990 ;136 :12 15-22(CD20)
	T-cells	CD3 (panTcell) (CD4); (CD8)	clone CRIS-7 (antiCD3); IF, IHC,WB	DIANOVA	Jones et al., J Immunol 1993 ; 150 :5429-35
	dysplastic and neoplastic cervical cells	$p16^{INK4a}$	E6H4, D7D7	MTM	Klaes R., et. al. Int J Cancer. 2001 Apr 15;92(2):276-84

tumor cells	different cancer cell types	P53 (mutations)			Mendoza-Rodriguez CA, et al., Rev Invest Clin 2001 May-Jun;53(3):266-73
	adeno-carcinoma cells	CEA			Mistretta et al., Experientia. 1974 Oct 15;30(10):1209-10; Rogers et al., Eur J Cancer Clin Oncol. 1984 Oct;20(10):1279-86
	bladder cancer cells	NMP22, BTA			van der Poel HG et al., Curr Opin Urol,11,503-509, 2001
	lung cancer cells	PreproGRP			Hamid et al., Cancer, 63, 266-271, 1989, Pagani et al., Int. J. Cancer 47, 371-375, 1991
Prolifer-ation	all proliferating cells	PCNA Ki67	Pc10, IgG2a	Zymed	Waseem NH, Lane DP, J Cell Sci 1990:96:121 (PCNA) Cattoretti et al., J Pathol 1992: 168:357-63(Ki67)
Infect-ious agent	HPV 16	E6	BF 7, IgG1 (IHC and in diagnostic kits for cervical swabs)	Research Diagnostics Inc.	Iftner et al., J Virol. 1988 Oct;62(10):3655-61
		L1	CamVir-1, IgG2a (IP, W, IF, IHC)	Research Diagnostics Inc.	Browne L et al., J Gen Virol. 1988 Jun;69 (Pt 6):1263-73
	HPV 18	L1	RDI-HPV18-5A3, IgG1 (W, IHC)	Research Diagnostics Inc.	Iftner et al., J Virol. 1988 Oct;62(10):3655-61

	HPV 6,11,18		RDI-HPVX-4C4	Research Diagnostics Inc.	Iftner et al., J Virol. 1988 Oct;62(10):3655-61 Gouillou et al., Am. J. Surg. Pathol., 1991

According to the present invention the normalization may comprise the determination of the presence of a number of (human) cells in question in a sample. This is a crucial aspect of the invention. In particular, embodiments false (especially false negative) results of tests can only be avoided, if the testing procedure verifies, that the test sample contains the materials (e.g. cells, tissues organisms etc.), that are necessary for performing the particular test. In various tests this will comprise ensuring, that the sample contains cells. In a wide range of embodiments of the invention the verification of the adequacy of the sample will not just comprise ensuring of the presence of cells, but will include the detection of the presence of cells of a distinct origin or of a special cell type.

Thus normalization may also comprise the determination of cells of particular origin such as e.g. cells from a particular organ or of a particular histological localization such as for example the detection of cells of distinct regions of epithelia, or of cells of connective tissue, cells originating from the basal lamina of a tissue or of cells of a heterologous origin, such as metastatic cells. This may be necessary in particular cases, because there might be cells, that under certain circumstances do express a marker, which might be used for the detection of a medically relevant condition, such as e.g. neoplasia or dysplasia, under certain normal conditions. Normalization as used according to the present invention may comprise the detection of the presence or absence and/or the level of any cell-types, that may possibly contribute to the total level of a particular marker selected to diagnose a medically relevant condition.

In one embodiment, the method may be applied for the detection of cervical lesions. Cervical lesion may comprise any kind of cervical dyplasia such as cervical cancers as defined above and its precursory stages. Markers and combinations thereof useful for this detection purpose are for example disclosed in WO0208764 and EP1217377, which documents shall be incorporated herein by reference. In this embodiment the test may be performed using any

suitable sample of cervical origin. The sample may for example comprise biopsies or microbiopsies of the cervix or swabs taken from the cervical region. Cervical swabs as used herein are samples that may for example be obtained using a suitable device such as a brush, a tampon, a spatula or the like, which is contacted with the uterine cervix during the sampling procedure. The sampling device may be any suitable device, which may be used in conventional testing performed by a physician or a self sampling device.

Promising molecular markers for enhancing the evaluation of cervical swabs are e.g. $p16^{INK4a}$, p14ARF, cyclin E, cyclin A, cyclin B, MN, her2/neu, mdm-2, bcl-2, EGF-Receptor, mcm-2, mcm-5, claudin-1, Markers indicative for Human papilloma virus infection, pRb, p53 etc. which might be used to detect dysplastic and neoplastic cells. Normalization according to the present invention for the purpose of analysis of cervical swabs may comprise the detection of the presence of human cells at all, the detection of cells of the cervical epithelium, the detection of the presence of endocervical as well as ectocervical cells and the detection of cells of endometrial origin. The endocervical epithelium is a glandular columnar epithelium. Cells originating from the endocervix may thus be identified by markers that are selectively expressed by columnar epithelial cells or by cells in glandular epithelia. The ectocervical epithelium is a squamous epithelium. Identification of ectocervical cells thus may be achieved by detection of markers characteristic for squamous epithelial cells. In certain embodiments, the detection of epithelial cells (comprising squamous as well as columnar epithelia) may be sufficient. In other embodiments, the differentiation of especially endocervical cells may be crucial. It is a crucial step to ensure the presence of ecto- and endocervical cells within the sample to ensure, that the specimen was taken at the cervical transformation zone, where most dysplasias and neoplasias arise. If there are no such cells, the sample is not adequate for the testing procedure, for it is prone to give false negative results. As $p16^{INK4a}$ may be expressed in normal endometrial cells normalization of the $p16^{INK4a}$ expression level in regard to the number of endometrial cells might be necessary.

To enable for reliable diagnosis the normalization furthermore may comprise the detection of the presence or absence of the named cellular components within the sample, and additionally the detection of the total level of a particular cell type or of the fraction, that a particular cell type contributes to the total number of cells within the sample.

Thus, in one embodiment the detected level of the $p16^{INK4a}$ protein may be normalized to the cytological conditions represented by the particular sample, so that one may state, if the detected level of the $p16^{INK4a}$ protein is indicative for cervical cells overexpressing $p16^{INK4a}$, or if there is an abundant number of endometrial cells present in the sample, thus mimicking the overexpression of $p16^{INK4a}$. In this respect normalization may comprise the determination of the quantity of endometrial cells within a cervical sample on the basis of a molecular marker. Comparing the level of e.g. $p16^{INK4a}$ as a marker for a medically relevant condition determined in a cervical sample to the quantity of endometrial cells assessed by means of molecular markers, one may state, whether the total amount of p16 may arise only from the endometrial cells present within the sample solution. Thus, false positive results in diagnosing cervical dysplasias overexpressing $p16^{INK4a}$ attributable to the presence of high levels of endometrial cells may be excluded. A quantity as used in the context of the present invention may refer to a quantitative or semi-quantitative assessment. This may e.g. comprise the assessment of a total number of cells or the assessment of a fraction with respect to the total number of cells. In certain embodiments of the invention the determination of a quantity may refer to the assessment of the fraction of an overall marker level that is contributed by a particular type of cells.

For the purpose of providing a normalization marker for the evaluation of cervical specimens, several normalization markers appear to be useful and may e.g. be selected from the following: Cytokeratins, E-Cadherins, Involucrin, Urokinase-like Plasminogen-activator, SCCA (Squamous cell carcinoma antigen), Catenins, (e.g. alpha-catenin, beta-Catenin, gamma-Catenin (Plakoglobin)), Ep-Cam.

Several candidates for normalization markers have been examined for their properties in characterization of cervical specimens. The results are given in Table 2 and Table 3.

Table 2

Name	Histology / Cytology	Clinical/Biochemical Data	Literature
UPA-1 (Urokinase-type Plasminogen-Activator; Swissprot Accession P00749; also known as EC 3.4.21.73, U-plasminogen activator uPA)	Cervical tissue Normal epithelium showed presence of both t-PA and u-PA immunoreactivity only in the superficial cellular layer, whereas in preinvasive lesions they were present in all layers.	↑ In cervix CA	Horn LC Aust N Z J Obstet Gynaecol, 2002 Larsson G Thromb. Haemost. 1987
PAI-1 (Plasminogen-Activator Inhibitor 1; Swissprot Accession P05121; also known as PAI-1 Endothelial plasminogen activator inhibitor PAI; Isoforms: PAI-2 P05120 and PAI-3 P05154)	Cervical tissue Normal epithelium showed presence of both t-PA and u-PA immunoreactivity only in the superficial cellular layer, whereas in preinvasive lesions they were present in all layers.	↑ In cervix CA positive prognostic marker	Horn LC Aust N Z J Obstet Gynaecol, 2002 Larsson G Thromb. Haemost. 1987
Involucrin (Swissprot Accession P07476)	Only squamous epithelia, no columnar cells; immature and mature squamous metaplastic cells. In normal epidermis, it is first expressed in the upper spinous layers, and in keratinocyte cultures it is expressed by all cells that have left the basal layer.	Involucrin expression is abnormal in squamous cell carcinomas and premalignant lesions, and is reduced in severe dysplasias of the larynx and cervix. Marker for terminal differentiation.	Shirley A, Human Pathology, 2001 de Boer et al., 1999, Am J of Pathol, 155:505-515 Nair SA, Pathobiology, 1996
gamma-Catenin (Swissprot Accession Q86W21; also known as Plakoglobin; e.g. Epitope: C-Terminus; AA553-738)	Squamous epithelia	High in normal cervical epithelium at cell-cell-boundaries. Moderate reduction in high grade SILS	de Boer et al., 1999, Am J of Pathol, 155:505-515

Alpha-1 Catenin (Swissprot Accession P35221; also known as Cadherin-associated protein Alpha E-Catenin)	Squamous epithelia	High in normal cervical epithelium at cell-cell-boundaries Strong reduction in high grade SILS	de Boer et al., 1999, Am J of Pathol, 155:505-515
Alpha-2 Catenin (Swissprot Accession P26232; also known as Alpha-Catenin related protein Alpha N-Catenin)			
beta-Catenin (Swissprot Accession P35222 also known as PRO2286)	Squamous epithelia	High in normal cervical epithelium at cell-cell-boundaries Strong reduction in high grade SILS	de Boer et al., 1999, Am J of Pathol, 155:505-515
Desmoplakin (Swissprot Accession P15924; also known as DP 250/210 kDa paraneoplastic pemphigus antigen)	stratified epithelia, simple epithelia, including glands, urothelium, thymic reticular epithelium, hepatocytes, intercalated disks of myocardium and arachnoid cells of meninges suprabasal layers of cervix (Superficial cells largely negative)	↓ in HSIL area	de Boer et al., 1999, Am J of Pathol, 155:505-515

↓: down regulated; ↑: up regulated;

Table 3

Marker	Histological testing	Cytological testing	Western Blot Analysis (clinical samples were freshly lysed with MTM buffer)
E-Cadherin (Swissprot Accession P12830; also known as Uvomorulin, Cadherin-1, CAM 120/80; e.g.epitope: C-Terminus; AA735-883)	Squamous epithelia, (Parabasal, intermediate cells) no columnar epithelia	Parabasal, intermediate cells, no columnar cells	Only weak signal for HT-29. All clinical samples are negative
p120 Swissprot Accession O60716; p120 catenin, p120(ctn), Cadherin-associated Src substrate, CAS, p120(cas); e.g.epitope: C-Terminus; AA790-911)	Squamous epithelia, (Parabasal, intermediate cells) also very strong in columnar epithelia	Very strong staining of parabasal, intermediate cells strong columnar cells	Only negative control (lymphocytes) and positive control (C4.1) positive
gamma-Catenin Swissprot Accession Q86W21; also known as Plakoglobin; e.g. epitope: C-Terminus; AA553-738)	Squamous epithelia, (Parabasal, intermediate cells) no columnar epithelia, total epithelium is stained indysplasia	Very strong staining of parabasal, intermediate cells no columnar cells	Double bands (82/95 kD) in 60 % of samples (9/15); after acetone precipitation of 150 µl of samples: 87% (13/15) positive
Ep-Cam (Tumor-associated calcium signal transducer 1, Swissprot Accession P16422; also known as Major gastrointestinal tumor-associated protein, GA733-2, Epithelial cell surface antigen, Epithelial glycoproteins, EGP, Adenocarcinoma-associated antigen KSA KS 1/4 antigen Cell surface glycoprotein Trop-1)	strong columnar epithelia, at very high concentrations rather unspecific (cytoplasmic) staining of squamous epithelia, (Parabasal, Intermediate Cells)	strong columnar cells, at very high concentrations rather unspecific (cytoplasmic) staining of squamous epithelia, (Parabasal, Intermediate Cells)	

Involucrin (Swissprot Accession P07476)	Strong staining of squamous epithelia, (Parabasal, Intermediate Cells) and columnar epithelia; unspecific staining of stromal cells;	All cells and structures positive	

The markers for normalization may for example be applied as markers indicative of the presence of specific cell differentiation patterns such as e.g. terminal differentiation or differentiation as specific epithelial cells. In certain embodiments, normalization markers may be marker molecules characteristic for squamous epithelial cells e.g. indicative for the presence of ectocervical cells in a cervical sample. Suitable markers may comprise e.g. CK13, E-Cadherin, gamma-Catenin, or Involucrin. In another embodiment the markers may be characteristic for the presence of columnar epithelial cells indicating the presence of endocervical cells in the specimen. Suitable markers comprise: Ep-Cam, CK18, CK8.

In certain embodiments, normalization may comprise the detection of epithelial cells generally; in these cases any marker suitable for the detection of epithelial cells may be employed. Markers may be for example those given in Tables 2 and 3.

In yet another embodiment of the invention, the method disclosed herein may be used for the detection of disorders of the respiratory tract. In the diagnosis of small cell lung cancer detection of neuron specific enolase (NSE) is one of the employed markers. Samples of tumor specimens are yielded by bronchoscopy with collection of cells by means of brushes or bronchoalveolar lavages. Since NSE is also expressed in few normal cells within the lung, the level of NSE expression detected in the dissolved sample has to be set in relation to the normalisation maker (for example actin) for detection of the amount of cells present within the sample.

A third embodiment of the present invention is the detection of lesions of the gastrointestinal tract, e.g. colorectal lesions from stool samples. In this case the origin of indicative nucleic acids and/or polypeptides detectable in stool samples may be crucial for the assessment of diagnosis. According to the present invention, it is possible to determine the origin (cell types/organism) of the employed marker molecules. Thus false results based e.g. on

the detection of marker molecules originating from foodstuff ingested by individuals rather than from lesion of the mucosa of the gastrointestinal tract may be eliminated. Furthermore artefacts produced by the presence of traces of markers from the blood circulation, or originating from swallowed sputum etc. may be eliminated using the methods disclosed herein.

Another aspect of the present invention is a testing kit for performing the method according to the present invention. The kit may be for example a diagnostic kit, an analytical kit or a research kit.

The term kit as used according to the present invention may comprise kits as well as diagnostic devices. The kits or devices may e.g. be designed for ELISA (e.g. sandwich, competitive, non-competitive, etc.), EIA (competitive, non-competitive, etc.) RIA tests, bead based test systems, lateral flow assays, flow through assays, strip test assays, dip stick assays, or any other known laboratory-, bench top- or point of care-testing format. A kit according to the present invention may in certain embodiments comprise in-vitro diagnostic devices for performing diagnostic tests. In vitro-diagnostic devices may e.g. be ELISA devices of any kind known to those of skill in the art. These devices comprise devices for sandwich ELISA formats, for competitive ELISA formats and any other ELISA formats. In another embodiment the in-vitro diagnostic device may be a lateral flow assay device, or a flow through assay device e.g. employing at least one reagent binding to a marker characteristic for a medically relevant condition and one reagent binding to a normalization marker, both fixed to a solid phase. Such devices may employ various mechanisms for visualization of the test result. In certain embodiments the tests may employ secondary detection reagents directed against the marker molecules coupled to detectable moieties. The detectable moieties may comprise colloidal gold, (coloured) latex particles and others.

In yet another embodiment the in-vitro diagnostic test device may be a flow through assay device based on capillaries or on porous members (such as membranes, beads or other three dimensional arrangements of porous substances). Depending on the embodiment the size of pores or capillaries need to be adjusted to ensure optimal flow conditions.

A kit according to present invention may comprise

a) reagents for the detection of the marker molecules,

b) the reagents and buffers commonly used for carrying out the detection reaction, such as buffers, detection-markers, carrier substances and others,

c) one or more markers and/or samples representative for medically relevant conditions to be diagnosed for carrying out positive and/or control reactions, and

d) one or more normalization marker samples for carrying out a positive and/or control reaction.

The test kit may optionally include a lysis buffer for solublization of the raw sample. Generally the lysis buffer may be any suitable solvent known to those of skill in the art. The lysis buffer for use in the kit may for example be aqueous solutions of chaotropic agents such as e.g. urea, GuaSCN, Formamid, of detergents such as anionic detergents (e.g. SDS, N-lauryl sarcosine, sodium deoxycholate, alkyl-aryl sulphonates, long chain (fatty) alcohol sulphates, olefine sulphates and sulphonates, alpha olefine sulphates and sulphonates, sulphated monoglycerides, sulphated ethers, sulphosuccinates, alkane sulphonates, phosphate esters, alkyl isethionates, sucrose esters), cationic detergents (e.g. cetyl trimethylammonium chloride), non-ionic detergents (e.g. Tween 20, Nonidet P-40, Triton X-100, NP-40, Igepal CA-630, N-Octyl-Glucosid) or amphoteric detergents (e.g CHAPS, 3-Dodecyl-dimethylammonio-propane-1-sulfonate, Lauryldimethylamine oxide) and/or of alkali hydroxides such as e.g. NaOH or KOH.

Examples of Lysis Buffers are given in Table 4.

Table 4

Lysis buffer	**Solubilization of $p16^{INK4a}$ in Western blot**	**compatibility with Elisa**
Detergents:		
0.1-1% SDS	+	+/-
0.2-3% SDS	+	< 0.5 %
0.2-3% DOC	++	+/-
0.1-1% n-Octylglycoside	+	yes
0.1-3% Triton x-100%	+	yes
0.1-1% Chaps	+	nd
Detergent-Mix:		
RIPA (1%NP40, 0.5%DOC, 0.1%SDS, PBS) 40-100%	++	yes
SOX (0.5% DOC, 0.5% n-Octylglycoside) 40-100%	+	yes
mtm lysis buffer (3% Tritonx-100, 0.4 % SDS, PBS)	++	yes
Commerical lysis buffers:		
Dynal (Dynal, Oslo, Norway)	++	yes
M-PER/B-PER (Pierce, Rockford, IL)	++	yes

Miscellaneous:		
0.5-8 M urea in PBS	+++	Compatible< 2 M
Lämmli sample buffer	+++	no
10-80% DMSO	+++	no
10-80 % Formamide	nd	no
50-70% formic acid	++	no
PBS	+/-	yes
Citrate buffer pH 6.0	+/-	yes
500 mM NaCl in Phosphate buffer	+/-	yes

nd: not determined; +/-: poor; +: good; ++: very good; +++: excellent;

The lysis buffer may furthermore comprise one or more agents that prevent the degradation of components within the raw samples. Such components may for example comprise enzyme inhibitors such as proteinase inhibitors, RNAse inhibitors, DNAse inhibitors etc. The inhibitors may e.g. comprise proteinase inhibitors selected from the compositions given in Table 5. In certain embodiments the lysis buffer by the way of providing an inhibitor of degradation enables for detection of p16 in the sample. In certain embodiments the cyclin dependent kinase inhibitor p16 is degraded in the solubilized samples and may thus not be detected. This is especially true, if the samples are directly transferred to a lysing medium and stored therein for a certain period of time.

Table 5

Inhibitor	**Class of inhibited proteinase**	**concentration**	**Solubility in water**	**stability in water**
Aprotinin	Serine	0.6-2 μg/ml	Very good	good
Benzamidine	Serine	0.5-4 mM	good	good
Bestatin	Aminopeptidases	1-10 μM	good	good
Calpeptin	Cysteine	0.3-1 μM	good	good
Cystatin	Cysteine	1 μM	good	good
E-64	Cysteine	1-10 μM	good	good
EDTA	Metallo	0.5-5 mM	good	good
Elastatinal	Serine	0.5-2 μg/ml	poor	good
EST	Cysteine	20-50 μg/ml	bad	poor
Fetal calf serum	all classes	10%	good	good
Leupeptin	Serine/Cysteine	10-100 μM	good	good
a2-Macroglobulin	all classes	1 μM	good	good
NCO-700	Cysteine	0.5-100 mM	poor	poor

Pefabloc= AEBSF	Serine	0.2-10 μM	good	very poor
Pepstatin A	Aspartic	1 μM	bad	poor
PMSF	Serine	0.2-10 μM	bad	very poor
o-Phenanthroline	Metallo	1-10 mM	bad	poor

For stabilization purpose the lysis buffer may also comprise bulk protein (e.g. albumin such as bovine serum albumin or calf serum albumin or other bulk proteins) to compete in degradation with the sample proteins. The bulk proteins may e.g. be present in combination with proteinase inhibitors or may be added instead of proteinase inhibitors. In one embodiment the solvent may be selected to be compatible with the performance of the test (EIA, ELISA or strip test performance), so that solubilized samples may directly be applied to the test. Test as used in the context may comprise any procedure for detecting the presence or absence and/or the level of marker molecules.

The reagent for the detection of the marker molecules may include any agent capable of binding to the marker molecule. Such reagents may include proteins, (poly)peptides, nucleic acids, peptide nucleic acids (PNAs), glycoproteins, proteoglycans, polysaccharids or lipids.

The markers characteristic for medically relevant conditions and/or normalization marker samples for carrying out positive and/or negative controls may comprise for example nucleic acids in applicable form such as solution or salt, peptides in applicable form, tissue section samples, microorganisms or positive or negative cell-lines.

In one embodiment of the invention, the detection of the marker molecules is carried out on the level of polypeptides. In this embodiment the binding agent may be for example an antibody specific for the marker molecules or a fragments thereof. Furthermore binding agents may comprise antigen-binding fragments such as Fab fragments, single chain antibodies,

bifunctional hybrid antibodies, peptidomimetics containing minimal antigen-binding epitopes etc. Moreover the binding agent might be a lectin binding to a specific carbohydrate structure on the marker molecule.

In another embodiment of the test kit the detection of the marker molecules is carried out on the nucleic acid level. In this embodiment of the invention the reagent for the detection may be for example a nucleic acid probe or a primer reverse-complementary to said marker nucleic acid.

The following examples are given for the purpose of illustration only and are not intended to limit the scope of the invention disclosed herein.

EXAMPLES

Example 1: Specific immunohistochemical detection of endocervical and ectocervical epithelial cells in cervical sections

In order to evaluate markers indicating the adequacy of cervical swabs, cervical sections (fixed in 4 % formaldehyde solution and paraffin-embedded) were stained with antibodies directed against Cytokeratin 18 (marker for endocervical columnar epithelia) and Cytokeratin 10/13 (marker for ectocervical squamous epithelia). Figure 1 shows specific staining of endocervical epithelia with anti-Cytokeratin 18 antibody and specific staining of ectocervical epithelia with anti-Cytokeratin 10/13 antibody. The experiment was performed as follows:

Formalin-fixed, paraffin-embedded sections were deparaffinized in xylene bath for 5 min (step was repeated once), excess liquid was tapped off and slides were placed in 95-96% ethanol for 3 (±1) min, in 70% ethanol for 3 (±1) min (step was repeated once) and finally in distilled water for a minimum of 30 sec. For epitope retrieval, slides were placed in a Coplin jar and boiled for 40 min at 95-99°C in 10 mM Citrate buffer pH 6.0. Slides were allowed to cool down for 20 min (±1 min) at RT in this buffer. Slides were covered with Peroxidase-Blocking Reagent (3% H_2O_2; NaN_3 15 mM) and incubated for 5 (±1) min at RT. After 5 min washing in washing buffer, slides were incubated with primary antibodies (CK 10/13: DE-K13, 1:50, DAKO; CK 18: K18.7, 1 µg/ml, dianova) for 30 min. Thereafter, slides were rinsed with wash buffer and washed in wash buffer for 5 min at RT. Following 30 min incubation with

EnVision (ready to use anti-mouse horseradish peroxidase-complex; DAKO), slides were washed 3x5 min and incubated in DAB substrate for 10 min, counterstained with hematoxylin and mounted with Faramount mounting medium.

Using an antibody directed against cytokeratin 18 (CK18) in an immunohistochemical staining procedure, a positive reaction was detected in columnar epithelium of the endocervix (Figure 1A), whereas the squamous epithelium of the ectocervix showed no specific staining (Figure 1B). Immunohistochemical staining with an antibody directed against cytokeratin 10/13 (CK 10/13) showed no staining in the columnar epithelium of the endocervix (Figure 1C), whereas there is a strong staining of the squamous epithelium of the ectocervix (Figure 1D). So CK18 might be used as a specific marker for the detection of columnar epithelial cells of the endocervix and CK10/13 as a specific marker for squamous epithelial cells of the ectocervix.

Example 2: Western Blot analysis of solubilized samples from cervical swabs

In order to evaluate, whether western blot analysis of solubilized samples allows assessing diagnosis of cervical lesions, clinical samples with known diagnosis were subjected to an immuno-chemical analysis on the basis of marker molecules after lysis of the sample material.

The clinical material (cervical swabs) samples were analyzed by Standard Western Analysis as follows.

In brief, the clinical material was in a first step solubilzed by boiling (5min, 95°C) in Lämmli Protein Sample buffer (100 mM Tris pH.6.8, 2% SDS, 200mM DTT, 0.05% BpB) prior to sonification. In a second step, protein samples were resolved on a SDS-PAGE (12% Acrylamide) and subsequently transferred on a nitrocellulose membrane by tank blotting (Towbin et al., 1979, Proc Natl Acad Sci: 76:4350-4354). In a further step, the membranes were: blocked to prevent unspecific antibody binding (10% non fat dry milk in PBS) and subsequently incubated with the specific monoclonal mouse antibody (CK 8: 35ßH11, 1:100, DAKO; $p16^{INK4a}$: D7D7, 1:140, MTM Laboratories). The binding of the specific antibody was visualized by Horseradish Peroxidase conjugated secondary reagents (binding to the marker specific antibody) catalyzing photon emitting substrates.

Cytokeratin 8 (CK 8) was used as an endocervical cell specific marker, indicating the adequacy of the sample collection in the present experiments. The cyclin dependent kinase inhibitor $p16^{INK4a}$ was used as specific disease related marker.

The results of the present experiment are given in Figure 2. The numbers 1 to 4 refer to samples (cervical swabs) obtained from individual patients. Immunoblot detection was performed using specific antibodies directed against cytokeratin 8 (CK8) and specific antibodies directed against $p16^{INK4a}$ (p16). The samples of patient 1, 2, and 3 show no signal for $p16^{INK4a}$. This indicates that no dysplastic cervical cells were present in these samples. The sample of patient 4 shows a strong signal for $p16^{INK4a}$. This indicates that dysplastic cervical cells were present in the sample. The upper bands show the specific signals for cytokeratin 8. In sample 1,3, and 4 cytokeratin 8 can be detected, whereas in for sample 2 no signal can be seen. This indicates that endocervical columnar cells were present in samples 1, 3, and 4, and absent in sample 2. As the presence of endocervical columnar epithelial cells is one of the parameters for the adequacy of cervical swabs, sample 2 is considered inadaequat and no diagnostic conclusions can be drawn from the negative result of the $p16^{INK4a}$ detection. Samples 1, 3, 4 are considered adequate. So based on the negative signal of sample 1 and 3 for $p16^{INK4a}$ it could be concluded, that these patients had no cervical dysplasia. Sample 4 showed a positive signal for $p16^{INK4a}$, indicating the presence of a dysplastic cervical lesion in this patient.

The parallel cytological analysis of the swabs indicated a normal cellular composition for woman 1 and 3. In women 2, no diagnosis due to sparse cellular material could be obtained. In woman 4, a high-grade dysplasia was diagnosed. Note that the upper band (CK 8) refers to the endocervical cell specific normalization marker Cytokeratin 8, indicating the adequacy of the sample collection. The lower band indicates the specific disease related marker $p16^{INK4a}$. The blot shows for patient 4 a positive signal for $p16^{INK4a}$ consistent with a high-grade cervical dysplasia. Samples of patient 1 and 3 show only the CK 8 specific band, indicating proper sample collection, but no disease related marker ($p16^{INK4a}$) consistent with a normal, healthy cervical epithelium. The sample of patient 2 shows no CK 8 signal, consistent with the low cell number in this sample, so no diagnostic conclusion can be drawn from the negative signal for $p16^{INK4a}$.

Example 3: Western blot and ELISA analysis to demonstrate sample adequacy

To evaluate, whether results of solution based analysis differing from diagnosis of samples may be due to inadequacy of sample, Western blot analysis of cervical swabs of four different patients with ascertained diagnosis (high-grade cervical intraepithelial neoplasia according to the cytological diagnosis of Pap IVa and Pap IVb) was performed. Antibody against $p16^{INK4a}$ was used to indicate presence of dysplastic cells, whereas antibodies against CK18 and CK10/13 were used to demonstrate adequacy of the sample.

Western blot analysis was performed as follows: Patient samples were collected with a cervical brush and directly lysed in Laemmli Sample Buffer (2% SDS, 60mM Tris pH.6.8, 0.01%, 100 mM DTT) for 5 min at 95°C ($1x10^7$ cells/ml) with subsequent sonification (5x5sec pulses, maximum intensity). Lysates were centrifuged for 12 min at 16,600xg) in a microcentrifuge and supernatant was transferred into a new tube. Precast 4-20% linear gradient Acrylamide gels (Criterion System, Bio-Rad) were loaded with 10µl (10^5 cells) of whole cell extracts and proteins were separated at 25mA constant current for 45 min. Proteins were transferred from the gel to Hybond ECL Nitrocellulose membrane (Amersham) by standard tank blotting using the Bio Rad Criterion Blotter (15 min at constant 100 Volt and subsequently 45min at constant 50 Volt). Nitrocellulose-membrane was stained for 5 min in Ponceau S solution to assure protein transfer. Ponceau S solution was removed by 2x10 min washes in PBS. For immunodetection, blots were blocked over night in blocking buffer (10% milk powder in PBS with 0.1% Tween-20). Primary antibodies were incubated at dilutions according to the manufacturer in blocking buffer for 1 h at RT with agitation (CK18: MAB 3236), 1:1000, CHEMICON; CK 10/13: DE-K13, 1:500, DAKO, $p16^{INK4a}$: D7D7, 1:140, MTM Laboratories). After 6 washes for 10 min with PBS/0.1% Tween-20, blots were incubated with rabbit anti mouse-HRP, (DAKO, diluted 1:5,000 in blocking buffer) for 1 h at RT. After 6 washes for 10 min with PBS/0.1% Tween-20, membranes were incubated for 5 min in substrate solution (Super Signal West Femto Maximum Substrate, Pierce), wrapped in a plastic envelope and exposed to an x-ray film for 1-5 min. Finally, x-ray films were developed, fixed, dried and documented with an imaging system (Bio-Rad). The same samples were used to perform

ELISA analysis for $p16^{INK4a}$, CK 10/13, CK18. The detected signals and results were the similar to the Western blot analysis and the same conclusions were drawn.

The ELISA analysis was performed as follows: Flat bottom 96 well plates (MaxiSorb; Nunc) were coated with capture antibody ($p16^{INK4a}$: MTM-E6H4, 2µg/ml in PBS, MTM Laboratories; CK10: MS481P1ABX, 2µg/ml, dianova; CK18: K18.7, 2 g/ml, dianova; 50µl/well) over night at 4°C. Plates were washed 6x with PBS/0.1% Tween-20 and blocked with Superblock buffer (Pierce). Solubilized protein extract from cervical swabs were dissolved in incubation buffer (PBS, 3% Superblock, 0.1% Tween20), and added in triplicates to each well. After 1 h incubation at RT, plates were washed 6x with PBS/0.1% Tween-20 and incubated with biotinylated detection antibody ($p16^{INK4a}$: MTM-D7D7 (0.2 µg/ml, MTM Laboratories, CK10: MS481-BO, 200 µg/ml, dianova; CK18: MS142-BO, 200 µg/ml, dianova; in incubation buffer) for 1 h at RT. Following 6x washes with PBS/0.1% Tween-20 TMB, 50 µl of Streptavidin-coated Alkaline Phosphatase (1:1000 dilution; Dianova) was added for 30 min. Thereafter, plates were washed 6x with PBS/0.1% Tween-20 and 100 µl of p-nitrophenyl phosphate substrate (PnPP; dissolved in diethanol amine buffer) were added to each well. OD 405 nm (620 nm reference wavelength) was measured with an ELISA reader (Tecan) after 30 min, 1h and 2 hrs. The present example shows, that the sandwich ELISA format exhibits sensitivity, which is suitable for the use in the methods according to the present invention. For use in the method disclosed herein the sandwich ELISA format as described in this example may be applied to multiple marker molecules, such as markers for normalization/adequacy and markers characteristic for medically relevant conditions.

Samples of four patients with high-grade cervical dysplasias (see Diagnosis) were analysed using western blot analysis (upper panel of figure). For the left blot immunoblot detection was performed using antibodies specific for β-actin and $p16^{INK4a}$, for the middle blot antibodies specific for cytokeratin 10/13 and for the right blot antibodies specific for cytokeratin 18 were used. ß-actin, CK18, and CK10/13 were used as markers demonstrating the adequacy of the sample. ß-actin indicates the presence of any cells, CK10/13 the presence of ectocervical squamous cells, CK 18 the presence of endocervical columnar cells.

As shown in Figure 3, for the samples of patient 1 and 2, the immunoblot detections show positive signals for all the applied adequacy markers (CK10/13, CK 18, ß-actin) and for

the marker ($p16^{INK4a}$) indicative of dysplastic cells. Samples 3 and 4 were negative for $p16^{INK4a}$ bands in Western blot. However, in these cases the ß-actin and the two cytokeratin markers showed an extremely weak (patient 3, ß-actin) or negative (patient 4, all markers; patient 3, CK markers) signal in the Western blot analysis. So no diagnostic conclusion can be drawn from the negative signal for $p16^{INK4a}$.

The lower panel of this figure shows the results of ELISA analysis. Positive signals for the adequacy markers (CK10/13, CK 18) were detected for the sample of patient 1 and 2, whereas for the samples of patients 3 and 4 no signals for CK10/13 and CK 18 was seen. So the ELISA analysis results resemble the Western blot analysis results and the same conclusions can be drawn.

Example 4: Western blot analysis of different samples of pulmonary origin

In order to evaluate, whether Western blot analysis of solubilized samples allows to assess diagnosis of pulmonary lesions, clinical samples with known diagnosis were solubilized and subjected to an immuno-chemical analysis on the basis of marker and normalization molecules.

The clinical samples (cells collected by brushing or bronchoalveolar lavage) were analyzed by Standard Western Analysis as follows. Cells from bronchoalveolar lavage were pelleted by centrifugation (5 min, 1000 rpm) and the pellet was dissolved in Lämmli Protein Sample buffer (100 mM Tris pH.6.8, 2% SDS, 200mM DTT, 0.05% BpB). Cells obtained by brushing were dissolved directly in Lämmli Protein Sample buffer (100 mM Tris pH.6.8, 2% SDS, 200mM DTT, 0.05% BpB). The material was boiled (5min, 95°C) prior to sonification. In a second step aliquots of the protein samples were resolved in duplicates on a SDS-PAGE (12% Acrylamide) and subsequently transferred on a nitrocellulose membrane by tank blotting (Towbin et al., 1979, Proc Natl Acad Sci;76:4350-4354). In a further step the membranes were blocked to prevent unspecific antibody binding (10% non fat dry milk in PBS) and subsequently one membrane was incubated with specific monoclonal mouse antibodies against NSE (DAKO Germany, clone BSS/NC/VI-H14, mouse monoclonal, dilution 1:1000;) and one membrane was incubated with the normalization marker actin (ICN, USA, clone C4, mouse

monoclonal, dilution 1:400). The binding of the specific antibody was visualized by Horseradish Peroxidase conjugated secondary reagents (binding to the marker specific antibody) catalyzing photon emitting substrates.

In the bronchoalveolar lavages of patients with known small cell lung cancers high levels of NSE in comparison with the expression levels of actin was detected, whereas in patients without tumor hardly any NSE could be detected, the actin level however was comparable to the level of the cancer patients. (Data not shown)

The results indicate, that a normalization of the solution based testing procedure according to the method presented herein enables for assessing diagnosis of diseases without relying on morphological information

Example 5: Detection of Cervical Intraepithelial Neoplasia in an ELISA test format

34 cervical swabs provided in lysis buffer have been subjected to ELISA based detection of overexpression of cyclin dependent kinase inhibitor $p16^{INK4a}$ in solutions prepared from the cells contained in the swabs. The ELISA testing was performed as follows:

(A) Cell Lysis

Cervical swab brushes are given into 15 ml vessels, containing 2 ml of mtm lysis buffer. Cervical cells present in the brush are lysed for at least 20h. The lysates of the cervical swab samples are then transferred in 2 ml tubes and are centrifuged at 4°C (15 min at 28.000 x g (16.600rpm Highspeed Centrifuge JEC Multi RF)); Supernatant is transferred to a fresh tube. As the case may be the supernatant may be stored at -20 °C.

(B) Performing the ELISA

Coating of ELISA-plates

Stock-solutions of $p16^{INK4a}$-specific antibody clone mtm E6H4, Ep-Cam specific antibody Ber-Ep4 and gamma-Catenin specific antibody clone 15 are diluted in PBS to give ready-to-use coating solution.

50µl of each ready-to-use capture antibody coating solution is added to ELISA plates. For coating, the plates are incubated overnight at 4°C.

Coating solutions are removed from the ELISA plates and the plates are rinsed using an automated ELISA washer as follows:

- 7 x 250µl washing buffer (0.1% Tween20 (v/v) in PBS)

after removing remnants of the washing buffer, 300µl blocking buffer (2% BSA in PBS) is added to each well. Plates are incubated for 1h on a rocking device at ambient temperature.

Incubation with samples

After removing the blocking buffer 100µl of the lysed cell sample is added to each well. Lysates of HeLa-cells are used as positive control for antibodies specifically detecting $p16^{INK4a}$ and gamma-Catenin; Lysates of HT29-cells are used as positive control for antibodies specifically detecting Ep-Cam;

For purpose of calibration of the test, different concentrations of recombinant p16 protein, recombinant gamma-Catenin and Ep-Cam (0 pg/ml, 50 pg/ml, 100 pg/ml, 200 pg/ml, 400 pg/ml, 800 pg/ml) are included in the test.

Samples are incubated for 1 h at room temperature.

Thereafter washing is performed on an automated ELISA washer as follows

- 7 x 250µl washing buffer. The remaining buffer is removed.

Incubation with detection antibody

Working solutions of biotinylated secondary antibodies (clone mtm D7D7 specific for $p16^{INK4a}$, clone A5B4 for Ep-Cam and clone MAB 2083 specific for gamma-Catenin) are prepared by dilution of stock solutions.

100µl of working solutions of biotinylated secondary antibodies are added to wells incubated with corresponding antigen and capture antibody. After incubation for 1h at RT, antibody solutions are removed and ELISA plates are washed by an automated ELISA washer

- 7 x with 250µl washing buffer.

Detection

Streptavidin-HRP-polymers (1mg/ml) are pre-diluted 1:10 (4µl +36µl incubation buffer); Final incubation solution is prepared by dilution 1:300 in incubation buffer (0,1% BSA in PBS) to a final concentration of 0,33 µg/ml.

100µl of this solution are added to each well and incubated for 1 h at RT.

Thereafter, the buffer is removed and the plates are washed manually with 200 µl washing buffer per well 5 times.

Substrate incubation

TMB-substrate is equilibrated to 25°C for 1h in the dark.

100µl of substrate solution is added to each well.

The ELISA plates are incubated at 25°C for exactly 15 min in the dark. Then the reaction is stopped by addition of 80 µl 2,5M H2SO4.

Within 5 min. after stopping the reaction OD 450 nm is determined. After evaluation of the results, each sample returns a value for the OD.

Evaluation of results

For sample adequacy, OD values of all samples for gamma-Catenin have to exceed a defined threshold value to prove proper sampling of a minimum of cells. Furthermore to ensure proper sampling a threshold for the OD value of Ep-Cam indicating the presence of endocervical cells has to be exceeded.

For detection of dysplastic cells, OD values for $p16^{INK4a}$ have to exceed a defined threshold value to prove the presence of a minimum of p16-positive dysplastic cells.

Results of this experiment are given in Table 6.

Table 6

No. of samples	$P16^{INK4a}$	Gamma-Catenin	Conclusion
3	+	+	Sample is adequate; $p16^{INK4a}$ indicates the presence of dysplastic cells
30	-	+	Sample is adequate; absence of detectable $p16^{INK4a}$ indicates absence of dysplastic cells
1	-	-	Sample is inadequate; re-sampling necessary

Comparison of OD values for $p16^{INK4a}$ and gamma-Catenin of 34 samples with corresponding threshold values revealed that 33 samples were adequate and could be further evaluated. From theses 33 samples, 30 samples were negative for $p16^{INK4a}$ and 3 were positive.

The ELISA results were compared to the diagnostic results of a Papanicolaou test (PAP test, cervical cytology) from the same patients. The cervical cytology were evaluated according to the Munich Classification II (1990). Pap II encompasses benign cells, cervicitis and metaplasia, Pap IV encompasses severe dysplasia and carcinoma in situ. It turned out that samples returning an OD for $p16^{INK4a}$ of greater than 0.9 in the ELISA correspond to samples, that are classified as dysplastic by the conventional cytological PAP test.

Applying OD 0.9 as threshold for the evaluation of the samples the ELISA results may be reported as follows.

Table 7

Diagnosis/ELISA results	ELISA positive for $p16^{INK4a}$	ELISA negative for $p16^{INK4a}$
Pap II	0	30
Pap IV	3	0
Not enough cells	0	1

The ELISA test is positive in all 3 samples (100%) from women having severe dysplasia and is negative in all 30 samples (100%) of women having no dysplasia. One sample only contained very few cells and therefore was excluded from evaluation, since sampling was inadequate.

The normalization of $p16^{INK4a}$ protein levels in solubilized patient samples with respect to a normalization marker characteristic for the presence of epithelial cells allows to assess diagnosis of dysplasias from the samples. The normalization in the present case allows especially to avoid false negative results due to inadequate sampling (for example total amount of patient material not sufficient to perform analysis, or the patient material is not taken at the correct anatomical location). The normalization is carried out in the testing format by applying a threshold value for the OD for the gamma-Catenin normalization marker determined in the ELISA above which the sample is to be classified as adequate. Below a certain threshold (corresponding to 200.000 squamous ectocervical cells) the sample does not contain an adequate amount of patient material. The use of a second normalization marker indicating the presence of endocervical cells provides further information about the adequacy of the sample. The normalization is carried out in the testing format by applying a threshold value for the OD for the Ep-Cam normalization marker determined in the ELISA above which the sample is to be classified as adequate. Below a certain threshold (corresponding to 2000 columnar endocervical cells) the sample does not contain an adequate amount of endocervical cells. (It must be understood that the threshold value applied in this example are adjusted to the particular reaction conditions. The value for the cells as well for OD may vary depending to the reaction conditions. Thus the values herein are intended to exemplify the conditions and not to limit the scope of the invention. Those of skill in the art know how an appropriate threshold value for a particular test format may be established.) The presence of endocervical cells provides the information that the swab or brush has had contact with the columnar epithelium of the endocervix and thus hints to a contact of the swab or brush with the transformation zone, where cervical dysplasia usually originates. In particular the detection of a certain amount of ectocervical cells (gamma-catenin) together with a certain amount of endocervical cells (Ep-Cam) provides with a high probability the information that the patient material was taken at the correct anatomical location (cervical transformation zone).

Using the threshold values evaluated in these experiments, cytological specimens of 300 patients were tested in the presented ELISA testing format. In this experiments the specimens identified as being dysplastic by cytological examination may also be identified as being dysplastic in the ELISA testing format.

Example 6: Detection of Cervical Intraepithelial Neoplasia in an ELISA test format.

The 34 cervical swabs as already used in Example 5 provided in lysis buffer have been subjected to ELISA based detection of overexpression of HPV E7 Protein and one adequacy marker in solutions prepared from the cells contained in the swabs. The ELISA testing was performed as follows:

(A) Cell Lysis

Cervical swab brushes are given into 15 ml vessels, containing 2 ml of mtm lysis buffer. Cervical cells present in the brush are lysed for at least 20h. The lysates of the cervical swab samples are then transferred in 2 ml tubes and are centrifuged at 4°C (15 min at 28.000 x g (16.600rpm Highspeed Centrifuge JEC Multi RF));Supernatant is transferred to a fresh tube. As the case may be the supernatant may be stored at -20 °C.

(B) Performing the ELISA

Coating of ELISA-plates

Stock-solutions of E7-specific antibody clone NM2 and gamma-Catenin specific antibody clone 15 are diluted in PBS to give ready-to-use coating solution.

50µl of each ready-to-use capture antibody coating solution is added to ELISA plates.

For coating, the plates are incubated overnight at 4°C.

Coating solutions are removed from the ELISA plates and the plates are rinsed using an automated ELISA washer as follows:

- 7 x 250µl washing buffer (0.1% Tween20 (v/v) in PBS)

after removing remnants of the washing buffer, 300µl blocking buffer (2% BSA in PBS) is added to each well. Plates are incubated for 1h on a rocking device at ambient temperature.

Incubation with samples

After removing the blocking buffer 100µl of the lysed cell sample is added to each well. Lysates of HeLa-cells are used as positive control for antibodies specifically detecting gamma-

Catenin; For purpose of calibration of the test, different concentrations of recombinant HPV 16 E7-protein, recombinant gamma-Catenin (0 pg/ml, 50 pg/ml, 100 pg/ml, 200 pg/ml, 400 pg/ml, 800 pg/ml) are included in the test.

Samples are incubated for 1 h at room temperature.

Thereafter washing is performed on an automated ELISA washer as follows

- 7 x 250µl washing buffer. The remaining buffer is removed.

Incubation with detection antibody

Working solutions of biotinylated secondary antibodies (clone NM13 specific for HPV16 E7 protein and clone MAB 2083 specific for gamma-Catenin) are prepared by dilution of stock solutions.

100µl of working solutions of biotinylated secondary antibodies are added to wells incubated with corresponding antigen and capture antibody. After incubation for 1h at RT, antibody solutions are removed and ELISA plates are washed by an automated ELISA washer

- 7 x with 250µl washing buffer.

Detection

Streptavidin-HRP-polymers (1mg/ml) are pre-diluted 1:10. (4µl +36µl incubation buffer); Final incubation solution is prepared by dilution 1:300 in incubation buffer (0,1% BSA in PBS) to a final concentration of 0,33 µg/ml.

100µl of this solution are added to each well and incubated for 1 h at RT.

Thereafter, the buffer is removed and the plates are washed manually with 200 µl washing buffer per well 5 times.

Substrate incubation

TMB-substrate is equilibrated to 25°C for 1h in the dark.

100µl of substrate solution is added to each well.

The ELISA plates are incubated at 25°C for exactly 15 min in the dark. Then the reaction is stopped by addition of 80 µl 2,5M H2SO4.

Within 5 min. after stopping the reaction OD 450 nm is determined. After evaluation of the results, each sample returns a value for the OD.

Evaluation of results

For sample adequacy, OD values of all samples for gamma-Catenin have to exceed a defined threshold value to prove presence of a minimum of epithelial cells. (cf. Example 5)

For detection of dysplastic cells, OD values for HPV 16 E7 have to exceed a defined threshold value to prove the presence of a minimum of transformed cells. The threshold depends on the ELISA conditions applied and was set as OD 0,7 in our test format.

Comparison of OD values for HPV 16 E7, gamma-Catenin of 34 samples with threshold values revealed that the 33 samples proven to contain epithelial cells by means of detection of gamma-Catenin.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications could be made without departing from the scope of the invention.